

Available online at www.sciencedirect.com

SCIENCE @ DIRECT®

Biochimica et Biophysica Acta 1758 (2006) 413–418

<http://www.elsevier.com/locate/bba>

Enhanced cellular binding of concatemeric oligonucleotide complexes

Olesya N. Simonova*, Albina V. Vladimirova, Marina A. Zenkova, Valentin V. Vlassov

Institute of Chemical Biology and Fundamental Medicine SB RAS, 8, Lavrentiev Ave., Novosibirsk 630090, Russia

Received 28 October 2005; received in revised form 22 February 2006; accepted 28 March 2006

Available online 4 April 2006

Abstract

Interaction of oligonucleotides condensed into long concatemeric complexes with cancer cells was investigated. Pairs of 24- and 25-mer oligodeoxyribonucleotides were designed so that they could hybridize and form concatemeric structures. Pre-assembling of the oligonucleotides into concatemers considerably enhanced their ability to bind to human embryo kidney 293 cells and neuroblastoma IMR-32 cells as compared to free oligonucleotides. Efficiency of concatemers binding to the cells is improved with increase of the length and concentration of concatemeric complexes. The obtained results suggest incorporation of pharmacologically active oligonucleotides into concatemeric complexes as an approach to improvement of their cellular interaction.

© 2006 Elsevier B.V. All rights reserved.

Keywords: Oligonucleotide complex; Oligonucleotide delivery; Cellular uptake; Antisense oligonucleotide

1. Introduction

Oligonucleotide-based therapeutics – antisense oligonucleotides, ribozymes and siRNAs – open up possibilities to affect intracellular gene expression and to silence foreign genes of infectious agents. [1,2]. These gene-targeted therapeutics can find applications in therapy aimed at a variety of diseases. However, poor uptake of oligonucleotides by cells remains to be the main obstacle significantly complicating their clinical implementation. Considerable efforts have been made to develop effective delivery systems for oligonucleotides (for review see [3]). Currently used approaches include the liposome-based techniques, conjugation of oligonucleotide to lipid or peptide molecules [4,5], absorption on cationic polymers and nanoparticles [6,7]. Viral and nonendocytic delivery systems also have been tested. In spite showing high delivery efficiency viral vectors currently appear to be rather dangerous for clinical use because of possible stimulation of insertional oncogenesis, undesirable immune responses and toxicities of some viral gene products for host cells [8–12].

Nonviral gene delivery relies mainly on the complexes formed with cationic liposomes. Many lipoplex formulations have been studied (for review see [13]), but their in vivo activity is generally lower as compared to that of viral systems. High toxicity of some cationic liposomes provides limitation for wide-spread therapeutic application as well [14–16].

Straightforward approach to improving oligonucleotide cellular uptake could be recruiting of natural mechanisms providing transport of extracellular nucleic acids into cells. Nucleic acids are known to be naturally secreted and absorbed by cells in living organisms [17–19]. Uptake of nucleic acids by cells is commonly considered to be mediated by absorptive endocytosis and fluid phase endocytosis [20,21]. Many reports have been made on the involvement of cell surface receptors in nucleic acids transport [22–28]. Attempts to investigate the mechanism of oligonucleotide uptake and intracellular trafficking were made [29–31]. However, direct mechanism of the oligonucleotide transport by receptor-mediated process needs further investigation and to date, no successful research on delivery of single-stranded oligonucleotides using the proteins capable of nucleic acids binding have been published. At the same time, accumulation of oligonucleotides on the cell surface is claimed to assist their uptake [32]. Also, it was shown that nucleic acids can bind to lipid surface through magnesium ion bridges and that this interaction is stimulated by assembly of

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; PAAG, polyacrylamide gel; PAGE, polyacrylamide gel electrophoresis

* Corresponding author. Tel.: +7 383 3333761; fax: +7 383 3333677.

E-mail address: olesya.simonova@niboch.nsc.ru (O.N. Simonova).

short nucleic acids into supramolecular complexes [33,34]. These data suggested that supramolecular complexes of oligonucleotides capable of multiple interactions with lipid membranes and cell surface receptors should bind to the cells more efficiently than individual oligonucleotides. Indeed, it was found that aggregation of oligonucleotides improves their delivery efficiency in comparison to monomer oligonucleotides [33, 35–38].

In the present study, we used two antisense sequences to design oligonucleotides, which could spontaneously assemble into long concatemeric complexes. We investigated the interaction of formed concatemeric structures of antisense oligonucleotides with different human cancer cells. It was found that pre-assembling of oligonucleotides into concatemeric complexes considerably enhances their ability to bind to the cells. The obtained results suggest simple approach for improving delivery of oligonucleotide-based therapeutics into cells.

2. Materials and methods

2.1. Preparation of concatemers

Oligonucleotides E1 5'-GGA AGT CCA GCC CCA TGG ATG ATG-3', E2 5'-GGC TGG ACT TCC CAT CAT CCA TGG-3', G1 5'-AAT TCC ACT GTA ATA ATA GGC ATA C-3', G2 5'-TAC TGT GGA ATT GTA TGC CTA TTA T-3', Stopper E 5'-TTT TCA TCA TCC ATG G-3'; Stopper G 5'-TTC CGT ATG CCT ATT AT-3' were synthesized by phosphoramidite method and purified by HPLC (ICBFM SB RAS). The purity of oligonucleotides analyzed by electrophoresis in 20% PAAG/8 M Urea gel was 95–98%.

³²P-labeling of the oligonucleotides was performed using γ -³²P-ATP and T4-polynucleotide kinase as described in [39]. To obtain concatemer structures, corresponding oligonucleotide strands (first strand was ³²P-labeled) were incubated at 37 °C for 30 min in appropriate buffer or in DMEM medium in the absence of serum. The resulting mixture was analyzed in native 10% PAGE run at 4 °C. After electrophoresis, gels were dried and the images were visualized using Molecular Imager FX (BioRad) and counted by Quantity One software. To evaluate the size of the formed complexes, plasmid pUC18 restriction products (fragments from 36 to 2686 bp) or 100–1000 bp DNA size marker (Sigma) were run in parallel and visualized by StainsAll (Sigma) staining.

2.2. Cell culture and binding studies

Studies of oligonucleotide interaction with cells were carried out using human embryo kidney 293 and neuroblastoma IMR-32 cell lines. The cells were grown in DMEM supplemented with 10% fetal bovine serum in incubator with conditions set to 37 °C and 5% CO₂. The cells were plated in the 24-well culture plates (1 × 10⁵ cells per well) and allowed to adhere overnight. Radiolabeled concatemers were prepared in serum free DMEM medium as described above and added to the cells in 250 μ l volume. After incubation, the medium with

oligonucleotides was removed and cells were washed with 0.5 ml ice-cold phosphate buffered saline for 2 min, and then lysed in 4 M guanidine thiocyanate solution containing 25 mM sodium citrate and 0.5% N-laurylsarcosine. The radioactivity of lysed product was measured by scintillation counting, and resulting values were counted excluding average absorption on the plate. Results were evaluated as percentage of cell-bound ³²P-labeled oligonucleotide. The final data were represented as means of 3 or 4 independent measurements with standard deviations as error bars. Statistical significance between groups was analyzed by Student's *t*-test. Differences in cell binding for concatemers and single strand oligonucleotides resulting in *P* < 0.05 were considered to be statistically significant.

3. Results

3.1. Design and formation of oligonucleotide concatemeric structures

Two antisense oligonucleotides E and G previously used for downregulation of human *mdr1* gene expression were chosen to develop oligonucleotide pairs capable of forming concatemeric complexes [40,41]. In order to get stable duplex formation under physiological conditions (37 °C), initial oligonucleotide sequences were extended up to 24 or 25 nt. Pairs for 24- and 25-mer antisense oligodeoxyribonucleotides (E1 and G1) were designed so that the 3'-half sequence of the second strand was complementary to the 3'-half of the E1 or G1 to provide the formation of double-stranded DNA complex upon hybridization (Fig. 1). The principle of oligonucleotide assembly was based on the half-sliding complementarity of the first and the second strands sequences. Pairing of one E1 or G1 molecule with E2 or G2, respectively, was expected to yield 12 bp duplex with 12 unpaired nucleotides at 5'-end of each of the strands. These free halves of the strands can hybridize with complementary part of another oligonucleotide molecule and thus induce spontaneous extension of concatemer structure (see Fig. 1). As a result, long double-stranded DNA complexes containing nicks at every 12 bp in alternate strands could be formed. To regulate the length of concatemers, the stopper oligonucleotide molecules (Stopper E and Stopper G, respectively) were added to the mixture. Sequences of stopper oligonucleotides represent the shortened versions of the second strands of oligonucleotide pairs. Hybridization of stopper to the 3'-end of the oligonucleotides E1 or G1 tail prevents binding of the second strand oligonucleotide and arrests the extension of the complex (Fig. 1).

Concatemeric complexes of oligonucleotides E and G were produced by incubation of corresponding oligonucleotides at

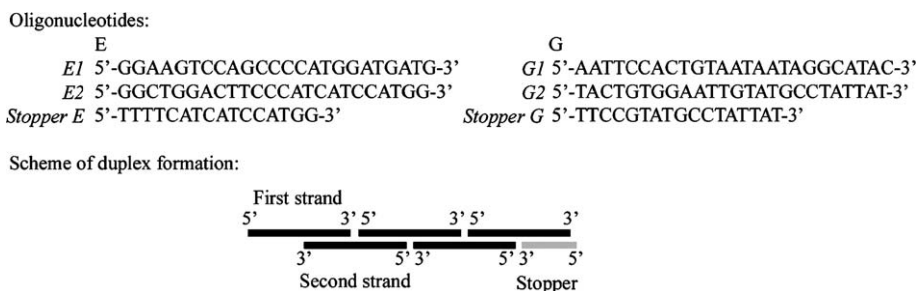


Fig. 1. Sequences of the oligonucleotides E and G and the concatemeric duplex formation.

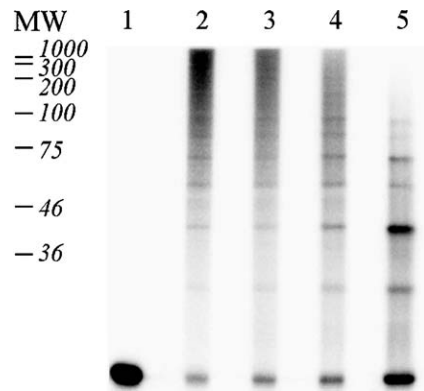


Fig. 2. Concatemeric complex formation in different buffer solutions (oligonucleotide G). Lane 1—5'-³²P-labeled oligonucleotide G1; lanes 2–5—concatemers formed by oligonucleotides G1 and G2 (1 μM each) in 50 mM Tris-HCl, pH 7.3, containing 200 mM KCl, 10 mM MgCl₂ (lane 2); in the same buffer in the absence of magnesium (lane 3); in 50 mM Tris-HCl, pH 7.3 (lane 4), and in H₂O (lane 5).

37 °C for 30 min without annealing step. After incubation, probes were mixed with loading buffer and immediately applied into the 10% native PAAG equilibrated at 4 °C. Concatemeric complexes were prepared in the following solutions: 1) 50 mM Tris-HCl, pH 7.3, 200 mM KCl, 10 mM MgCl₂; 2) 50 mM Tris-HCl, pH 7.3, 200 mM KCl; 3) 50 mM Tris-HCl, pH 7.3; 4) H₂O. The longest concatemers were formed in the solution containing 10 mM magnesium (Fig. 2).

We studied formation of the concatemeric duplexes at different concentrations of oligonucleotide strands in the presence and in the absence of the stopper oligonucleotides in the solution containing potassium and magnesium at physiological concentrations (Fig. 3). According to the results of gel-shift analysis, in the absence of stopper oligonucleotides pairs of oligonucleotides E and G form concatemeric duplexes up to

2000 bp in length. The effect of oligonucleotides concentrations on the concatemer formation was also investigated (Fig. 3, lanes 10–15). At equimolar concentrations of oligonucleotide strands in the reaction mixture, the main pool of the concatemers is represented by complexes above 200 bp in size. Excess of one of the strands results in formation of shorter duplexes. These results are in agreement with the previously published data on the nicked DNA duplexes formation, according to which equimolar stoichiometry of oligonucleotide components is preferable for the formation of longer nicked double-stranded structures [42].

As it could be expected, addition of the stopper resulted in formation of short complexes. Association of E oligonucleotides revealed non-uniform formation of concatemeric structures resulting in different intensity of bands seen in Fig. 3, lanes 6, 7, 12–15. This effect can be explained by more effective hybridization of one of the 12 nt oligonucleotide halves to its complementary sequence due to the asymmetry in G:C pairs distribution in the E oligonucleotide sequence.

3.2. Interaction of oligonucleotide concatemers with cells

To explore the influence of oligonucleotides association into concatemeric complexes on their interaction with eukaryotic cells ³²P-labeled concatemers were incubated with different human cancer cell lines. Experiments with 293—human embryo kidney cells and IMR-32—neuroblastoma cells demonstrated enhanced cellular binding of oligonucleotides in concatemeric complexes (1 μM concentration in the medium) as compared to single-stranded oligonucleotides at the same concentration (Fig. 4). The data show reliable increase of binding (2–4 times) of concatemers E and G with 293 cells and concatemers G with IMR-32 cells as compared to single-stranded oligonucleotides, while the difference in concatemers

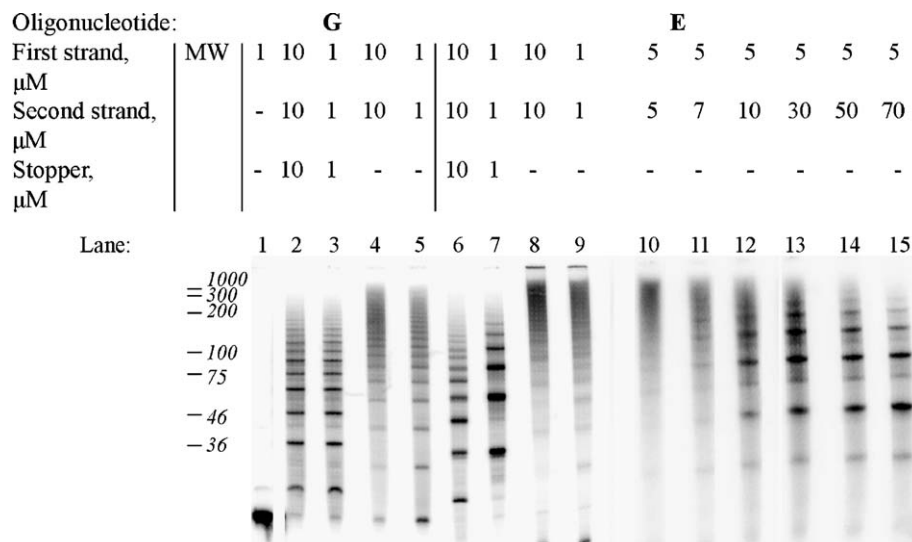


Fig. 3. Influence of strands concentrations on the length of concatemeric duplexes formed in solution containing 50 mM Tris-HCl, pH 7.3, 200 mM KCl, 10 mM MgCl₂. Lane 1—5'-³²P-labeled oligonucleotide G1; lanes 2, 3, 6, 7—concatemeric structures formed by E and G oligonucleotides in the presence of stoppers; lanes 4, 5, 8, 9—concatemeric duplexes formed in the absence of stoppers; MW—size marker. Lanes 11–15—formation of concatemers of oligonucleotides E under conditions of excess of the second strand—E2 (E1—³²P-labeled).

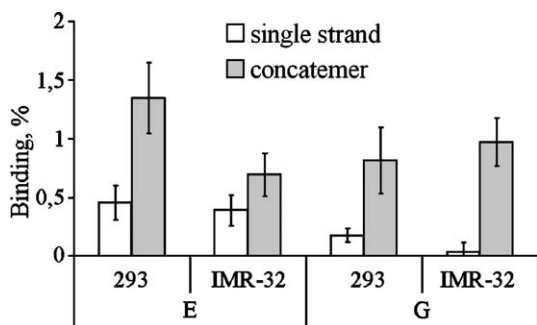


Fig. 4. Binding of oligonucleotides E1 and G1 and concatemers, formed by E1 and E2 or G1 and G2 oligonucleotides with 293 and IMR-32 cells. Cells were incubated with oligonucleotides for 3 h, the concentration of oligonucleotides was 1 μ M.

E and oligonucleotide E binding with IMR-32 cells was within the limits of standard deviation. In contrast, binding of concatemeric complexes with KB-3-1—epidermoid carcinoma and HeLa—ovarian carcinoma cells did not exceed the level of binding for single-stranded oligonucleotides (data not shown). Thus, the effect of enhanced binding for concatemeric complexes is cell type-dependent.

The influence of the concentration of concatemers and free oligonucleotides (oligonucleotide E) on the binding with 293 cells was investigated. Equimolar concentrations of oligonucleotide strands in the absence of the stopper were used. It is seen (Fig. 5A) that in contrast to monomer oligodeoxyribonucleotides, the concatemeric oligonucleotide complexes demon-

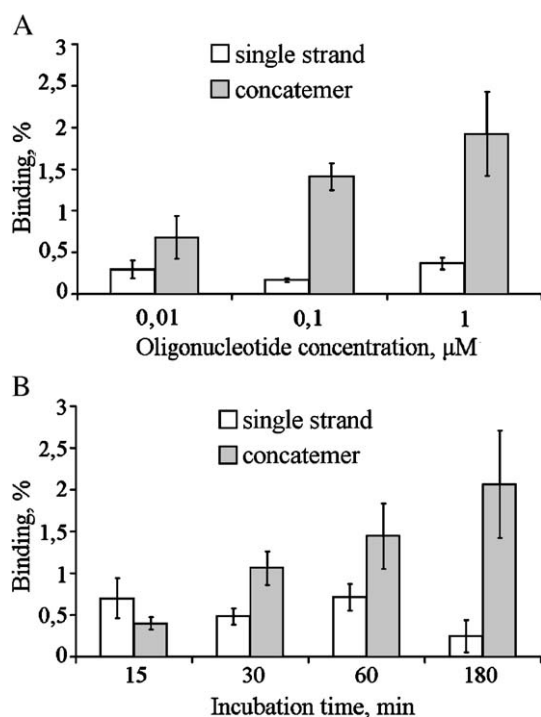


Fig. 5. Binding of oligonucleotide E1 and concatemers formed by equimolar mixture of oligonucleotides E1 and E2 (in the absence of the Stopper E) with embryo kidney 293 cells. (A) Concentration dependences of binding of the oligonucleotide E1 and corresponding concatemer complex after 3 h incubation with cells. (B) Kinetics of the oligonucleotide and concatemer binding to the cells. Oligonucleotides concentration in the medium was 1 μ M.

strate more efficient cellular binding at higher oligonucleotide concentrations within the interval 0.01–1 μ M.

Time course of oligonucleotides and concatemers (1 μ M concentrations of the strands) binding with 293 cells is shown in Fig. 5B. The results of the experiments revealed that binding of the free oligonucleotide is not increased after 30 min of incubation. In contrast, the amount of cell-bound oligonucleotides assembled into concatemeric structures is slowly increased during 3-h incubation, showing that the interaction of these complexes with the cells is characterized by slow kinetics (Fig. 5B).

The length of the concatemers formed upon hybridization of oligonucleotides was shown to depend on the oligonucleotide components concentration (Fig. 3). We compared efficiency of cellular binding of concatemeric complexes of different size formed by oligonucleotides E (Fig. 6). The most efficient binding was observed for the largest complexes formed when the strands were presented at equimolar concentrations. Cell binding of short concatemers, formed in the presence of stopper or under the excess of one of the strands, was comparable with the binding of single-stranded oligonucleotides or the difference was not statistically reliable. In 1 μ M equimolar solution of oligonucleotides E1 and E2 only a fraction of the oligonucleotides is involved in the complexes (Fig. 6B, lane 1). As it was shown that cellular binding efficiency of the single-stranded oligonucleotides even at higher concentration (Fig. 6, lane 6) is lower than binding of mixture containing concatemers, it can be concluded that the main contribution to the increased binding

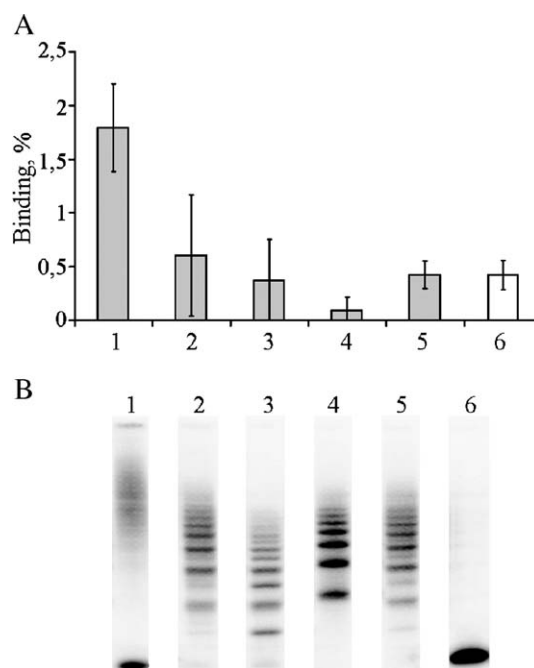


Fig. 6. (A) Influence of the size of concatemeric duplexes formed by oligonucleotides E1, E2 and Stopper E on their interaction with 293 cells. (B) Analysis of concatemer length (in the medium after incubation with the cells) by native PAAG electrophoresis, 32 P-label is located at 5'-end of E1. Lane 1—1 μ M E1 and E2; lane 2—1 μ M E1, E2 and Stopper E; lane 3—1 μ M E1 and E2, 2 μ M Stopper E; lane 4—5 μ M E1 and 30 μ M E2; lane 5—10 μ M E1, E2 and Stopper E; lane 6—10 μ M E1.

efficiency is due to the longer concatemeric complexes. The length of the concatemeric complexes was analyzed by gel electrophoresis prior and after the incubation with the cells. Long concatemers were shown to be stable for at least 3 h administration to the cells (see Fig. 6B).

4. Discussion

Aggregation of oligonucleotides facilitates their cellular binding and uptake. Therefore, we addressed the question whether the formation of concatemeric complexes improves the oligonucleotide binding by the eukaryotic cells. We designed concatemeric oligonucleotide structures built of half-slide complementary oligonucleotide pairs. Hybridization of overlapping oligonucleotide strands results in formation of double-stranded structure with periodic nicks in their backbone, which increase the flexibility of the formed long DNA duplex [42]. Due to efficient stacking between adjacent oligonucleotides the concatemer complex is thermodynamically more stable than the corresponding duplexes formed by oligonucleotides of the same length. Contiguous hybridization leads to partial removal of fraying at the adjacent oligonucleotide ends, and the effect of the direct stacking of the terminal base pairs in the duplex junction provides more auspicious conditions for hybridization of the next oligonucleotide molecule thus contributing to the stability of long concatemeric complexes [43–45].

The choice of oligonucleotides to perform the formation of concatemeric complexes follows several requirements. Initial sequence of the first strand is determined by the biological target sequence of the oligonucleotide. Free oligonucleotide halves should be approximately equal in length and melting temperatures (i.e., A:T/G:C pair distribution) to obtain uniform extension of the duplex. In our study we observed that misbalance in only one G:C pair led to noticeable change in the character of concatemeric duplex pattern especially at low concentrations of oligonucleotide components (Fig. 2). Self-complementarity and secondary structures should be avoided for both oligonucleotide strands. Moreover, possibility of undesirable sliding hybridization between oligonucleotides should be excluded because it might lead to alternative complexes formation (including bulges and gaps) and thus disrupt the appropriate extension of concatemeric duplex. In spite of this seeming complexity, the design of concatemeric oligonucleotides turns out to be easier as at least some of this requirements (particularly, lack of self-complementary structures) are inevitable for the choice of antisense sequences. Desired oligonucleotide also can be extended according to the target mRNA sequence to obtain more stable complexes. In any case, the efficiency of concatemeric complex formation and influence of such sequence extension on its biological activity should be verified individually.

We found that concatemeric oligonucleotide structures bind to 293 and IMR-32 cells considerably better than monomer oligonucleotides. Concatemers interaction with cells is more efficient at higher oligonucleotides concentration, which is not the case for the single-stranded oligonucleotides at the used

concentrations. Apparently, this phenomenon is a result of more efficient formation of long complexes at higher concentrations of oligonucleotide components in the medium.

The slow kinetics of concatemer binding to the cells can be explained by implying different mechanisms of absorption by cells for single-stranded oligonucleotides and concatemers. Considerable binding increase for the longest concatemer molecules consisting of many copies of assembling oligonucleotides suggests that oligonucleotide uptake in the supramolecular condensed form leads to the improvement of delivery efficiency. It is possible that the rise of local concentration of oligonucleotides provided by association into concatemers leads to the enhancement of penetration through the cell membrane. As we did not address the mechanism of the cell binding process, it remains questionable whether the effect of enhanced concatemer binding is due to formation of more condensed oligonucleotide particles on the cell surface or due to higher efficiency of interaction with cellular receptors. Cell type specificity of the phenomenon may support the concept of the involvement of cell surface proteins.

Concentration of oligonucleotides at the cell surface turns out to be the major factor determining efficiency of cellular uptake of oligonucleotides. The phenomenon of enhanced binding of concatemeric oligonucleotide structures by cells suggests an approach to improving cellular delivery of oligonucleotide therapeutics. This strategy could be applicable for antisense oligonucleotides and siRNA, which can fulfill the requirements to be assembled in concatemer complexes. Oligonucleotide concatemers are easy to prepare and were shown to be stable at physiological concentrations of salts and during incubation with cell culture. Incorporation of therapeutic oligonucleotides in concatemeric structures provides an additional advantage—protection of oligonucleotide molecules from cellular nucleases, which readily destroy single-stranded oligonucleotides. Whether this approach will provide enhanced oligonucleotide delivery to their intercellular targets and improve biological activity of oligonucleotide therapeutics remains to be investigated.

Acknowledgements

This work was supported by grants RFBR - 05-04-48985, RI-112/001/254 RAS programs “Molecular and Cellular Biology”, “Science to Medicine”.

References

- [1] J.B. Opalinska, A.M. Gewirtz, Nucleic-acid therapeutics: basic principles and recent applications, *Nat. Rev., Drug Discov.* 1 (2002) 503–514.
- [2] N.M. Dean, Functional genomics and target validation approaches using antisense oligonucleotide technology, *Curr. Opin. Biotechnol.* 12 (2001) 622–625.
- [3] S. Akhtar, M.D. Hughes, A. Khan, M. Bibby, M. Hussain, Q. Nawaz, J. Double, P. Sayyed, The delivery of antisense therapeutics, *Adv. Drug Deliv. Rev.* 44 (2000) 3–21.
- [4] S. Capaccioli, G. Dipasquale, E. Mini, T. Mazzei, A. Quttrone, Cationic lipid improve antisense oligonucleotide uptake and prevent degradation in cultured cells and human serum, *Biochem. Biophys. Res. Commun.* 197 (1993) 818–825.

- [5] D. Lochmann, E. Jauk, A. Zimmer, Drug delivery of oligonucleotides by peptides, *Eur. J. Pharm. Biopharm.* 58 (2004) 237–251.
- [6] H. Yoo, R.L. Juliano, Enhanced delivery of antisense oligonucleotides with fluorophore-conjugated PAMAM dendrimers, *Nucleic Acids Res.* 28 (2000) 4225–4231.
- [7] S. Bisht, G. Bhakta, S. Mitra, A. Maitra, pDNA loaded calcium phosphate nanoparticles: highly efficient non-viral vector for gene delivery, *Int. J. Pharm.* 288 (2005) 157–168.
- [8] T. Cartmell, T. Southgate, G.S. Rees, M.G. Castro, P.R. Lowengstein, G.N. Luheshi, Interleukin-1 mediates a rapid inflammatory response after injection of adenoviral vectors into brain, *J. Neurosci.* 19 (1999) 1517–1523.
- [9] S. Worgall, G. Wolf, E. Falk-Pedersen, R.G. Crystal, Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration, *Hum. Gene Ther.* 8 (1997) 37–44.
- [10] Y. Yang, F.A. Nunes, K. Berensi, E.E. Furth, E. Gönczöl, J.M. Wilson, Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy, *Proc. Natl. Acad. Sci. U. S. A.* 91 (1994) 4407–4411.
- [11] P.R. Lowenstein, E.E. Morrison, D. Brain, P. Hodge, C.M. Preston, P. Clissold, N.D. Stow, T.A. McKee, M.G. Castro, Use of recombinant vectors derived from herpes simplex virus 1 mutant tsK for short-term expression of transgenes encoding cytoplasmic and membrane anchored proteins in postmitotic polarized cortical neurons and glial cells in vitro, *Neuroscience* 60 (1994) 1059–1077.
- [12] O. Stone, A. David, F. Bolognani, P.R. Lowenstein, M.G. Castro, Viral vectors for gene delivery and gene therapy within the endocrine system, *J. Endocrinol.* 164 (2000) 103–118.
- [13] S.-A.L. Audouy, L.F.M.H. de Leij, D. Hoekstra, G. Molema, In vivo characteristics of cationic liposomes as delivery vectors for gene therapy, *Pharm. Res.* 19 (2002) 1599–1605.
- [14] J.D. Toussignant, A.L. Gates, L.A. Ingram, C.L. Johnson, J.B. Nietupski, S.H. Chen, S.J. Eastman, R.K. Scheule, Comprehensive analysis of the acute toxicities induced by systemic administration of cationic lipid: plasmid DNA complexes in mice, *Hum. Gene Ther.* 11 (2000) 2493–2513.
- [15] Y.K. Song, F. Liu, S. Chu, D. Liu, Characterization of cationic liposome-mediated gene transfer in vivo by intravenous administration, *Hum. Gene Ther.* 8 (1997) 1585–1594.
- [16] S. Loisel, C. Le Gall, L. Doucet, C. Ferec, V. Floch, Contribution of plasmid DNA to hepatotoxicity after systemic administration of lipoplexes, *Hum. Gene Ther.* 12 (2001) 685–696.
- [17] P. Laktionov, J.-E. Dazard, J. Piette, E. Vives, E. Rykova, V. Vlassov, B. Lebleu, Uptake of oligonucleotides by keratinocytes, *Nucleosides Nucleotides* 18 (1999) 1697–1699.
- [18] S.L. Loke, C.A. Stein, X.H. Zhang, K. Mori, M. Nakanishi, C. Subasinghe, J.S. Cohen, N.L. Neckers, Characterization of oligonucleotide transport into living cells, *Proc. Natl. Acad. Sci. U. S. A.* 86 (1989) 3474–3478.
- [19] E.S. Morozkin, P.P. Laktionov, E.Y. Rykova, O.E. Bryzgunova, V.V. Vlassov, Release of nucleic acids by eukaryotic cells in tissue culture, *Nucleosides Nucleotides Nucleic Acids* 23 (2004) 927–930.
- [20] V.V. Vlassov, L.A. Balakireva, L.A. Yakubov, Transport of oligonucleotides across natural and model membranes, *Biochim. Biophys. Acta* 1197 (1994) 95–108.
- [21] Y. Kimura, K. Sonehara, E. Kuramoto, T. Makino, T. Yamamoto, T. Kataoka, T. Tokunaga, Binding of oligoguanylate to scavenger receptor is required for oligonucleotides to augment NK cell activity and induce IFN, *J. Biochem.* 116 (1994) 991–994.
- [22] C. Beltinger, H.U. Saragovi, R.M. Smith, L. LeSauter, N. Shah, L. DeDonisio, L. Christensen, A. Raible, L. Jarett, A.M. Gerwitz, Binding, uptake and intracellular trafficking of phosphothioate-modified oligodeoxynucleotides, *J. Clin. Invest.* 95 (1995) 1814–1823.
- [23] J. Rappaport, B. Hans, J.B. Kopp, T.D. Copeland, I.A. Bruggeman, T.M. Coffman, P.E. Klotman, Transport of phosphorothioate oligonucleotides in kidney: implications for molecular therapy, *Kidney Int.* 47 (1995) 1462–1469.
- [24] M.A. Guvakova, L.A. Yakubov, I. Vlodavsky, J.L. Tonkinson, C.A. Stein, Phosphorothioate oligodeoxynucleotides bind to basic fibroblast growth factor, inhibit its binding to cell surface receptors and remove it from low affinity binding sites on extracellular matrix, *J. Biol. Chem.* 270 (1995) 2620–2627.
- [25] D.A. Geselowitz, L.M. Neckers, Bovine serum albumin is a major oligonucleotides-binding protein found on the surface of cultured cells, *Antisense Res. Dev.* 5 (1995) 213–217.
- [26] P. Hawley, J.S. Nelson, K.L. Fearon, G. Zong, I. Gibson, Comparison of binding of N3'-P5' phosphoramidate and phosphorothioate oligonucleotides to cell surface proteins of cultured cells, *Antisense Nucleic Acids Drug Dev.* 9 (1999) 61–69.
- [27] O.E. Shestova, Alu Andreeva, V.V. Vlassov, L.A. Yakubov, Transport of oligonucleotides-cell surface proteins complexes into cell nucleus, *Dokl. Akad. Nauk* 368 (1999) 264–267.
- [28] L.A. Yakubov, E.A. Deeva, V.F. Zarytova, E.M. Ivanova, A.S. Rytte, L.V. Yurchenko, V.V. Vlassov, Mechanism of oligonucleotide uptake by cells: involvement of specific receptors? *Proc. Natl. Acad. Sci. U. S. A.* 86 (1989) 6454–6458.
- [29] D.A. Geselowitz, L.M. Neckers, Analysis of oligonucleotides binding, internalization and intracellular trafficking utilizing a novel radiolabeled crosslinker, *Antisense Res. Dev.* 2 (1992) 17–25.
- [30] D. Nakai, T. Seita, T. Terasaki, S. Iwasa, Y. Shoji, Y. Mizushima, Y. Sugiyama, Cellular uptake mechanism for oligonucleotides: involvement of endocytosis in the uptake of phosphodiester oligonucleotides by a human colorectal adenocarcinoma cell line, HCT-15, *J. Pharmacol. Exp. Ther.* 278 (1996) 1362–1372.
- [31] V.V. Vlassov, M.V. Nechaeva, S.I. Baiborodin, O.E. Shestova, I.V. Saifonov, A.A. Koshkin, L.A. Yakubov, Oligonucleotides absorbed by a cell quickly accumulate in the nucleus, *Dokl. Akad. Nauk* 345 (1995) 123–126.
- [32] D. Luo, W.M. Saltzman, Enhancement of transfection by physical concentration of DNA at the cell surface, *Nat. Biotechnol.* 18 (2000) 893–895.
- [33] M.F. Yanze, W.-S. Lee, K. Poon, M. Piquette-Miller Jr., R.B. Macgregor, Cellular uptake of DNA frayed wires, *Biochemistry* 42 (2003) 11427–11433.
- [34] A. Vlassov, A. Khorova, M. Yarus, Binding and disruption of phospholipid bilayers by supramolecular RNA complexes, *Proc. Natl. Acad. Sci. U. S. A.* 98 (2001) 7706–7711.
- [35] X. Gao, L. Huang, Potentiation of cationic liposome-mediated gene delivery by polycations, *Biochemistry* 35 (1996) 1027–1036.
- [36] W.T. Godbey, K.K. Ku, G.J. Hirasaki, A.G. Mikos, Improved packing of poly(ethylenimine)/DNA complexes increases transfection efficiency, *Gene Ther.* 6 (1999) 1380–1388.
- [37] A. Astriab-Fisher, M.H. Fisher, R. Juliano, P. Herdewijn, Increased uptake of antisense oligonucleotides by delivery as double stranded complexes, *Biochem. Pharmacol.* 68 (2004) 403–407.
- [38] C. Wu, J. Castro, M. Motta, H.B. Cottam, D. Kyburz, T. Kipps, M. Corr, D. Carson, Selection of oligonucleotide aptamers with enhanced uptake and activation of human leukemia B cells, *Hum. Gene Ther.* 14 (2003) 849–860.
- [39] B. Perbal, *A Practical Guide to Molecular Cloning*, Academic Press, Inc., New York, 1984.
- [40] E.V. Kostenko, R.S. Beabealashvili, V.V. Vlassov, M.A. Zenkova, Secondary structure of the 5'-region of PGY1/MDR1 mRNA, *FEBS Lett.* 475 (2000) 181–186.
- [41] E.V. Kostenko, P.P. Laktionov, V.V. Vlassov, M.A. Zenkova, Down-regulation of PGY1/MDR1 mRNA level in human KB cells by antisense oligonucleotide conjugates. RNA accessibility in vitro and intracellular antisense activity, *Biochim. Biophys. Acta* 1576 (2002) 143–147.
- [42] T. Sarkar, C.C. Conwell, L.C. Harvey, C.T. Santai, N.V. Hud, Condensation of oligonucleotides assembled into nicked and gapped duplexes: potential structures for oligonucleotide delivery, *Nucleic Acids Res.* 33 (2005) 143–151.
- [43] D.V. Pyshnyi, E.M. Ivanova, The influence of nearest neighbours on the efficiency of coaxial stacking at contiguous stacking hybridization of oligodeoxyribonucleotides, *Nucleosides Nucleotides Nucleic Acids* 23 (2004) 1057–1064.
- [44] D.V. Pyshnyi, I. Pyshnaya, A. Levina, E. Goldberg, V. Zarytova, D. Knorre, E. Ivanova, Thermodynamic analysis of stacking hybridization of oligonucleotides with DNA template, *J. Biomol. Struct. Dyn.* 19 (2001) 555–570.
- [45] Z.A. Shabarova, N.G. Dolinnaya, S.I. Turkin, E.S. Gromova, DNA-like duplexes with repetitions. I. Properties of concatemer duplexes formed by d(T–G–C–A–C–A–T–G), *Nucleic Acids Res.* 11 (1980) 2413–2429.